Tumor Infiltrating Dendritic Cells Predict Treatment Response to Immmunotherapy in Patients with Metastatic Renal Cell Carcinoma

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Abstract. *Background: Although renal cell carcinoma (RCC) is considered to be an immunogenic tumor, the role of immunogenicity in this tumor for predicting treatment response has been little investigated. Patients and Methods: Resected RCC specimens from 25 patients who received cytokine treatment for metastases were investigated using immunohistochemistry for CD83+ or S100+ dendritic cells (DCs), CD8+ T-cells, HLA-DR+ tumor cells, CD68+ tumor associated macrophages, microvascular density and vascular endotherial growth factor. Results: Among the examined parameters, DCs status showed predictive value, that is, higher numbers of CD83+ or S100+ cells in tumors were associated with favorable treatment response. However, only higher CD83 status, which indicates mature and activated DCs, contributed to better survival (p=0.0339). Conclusion: Increased tumor infiltration of mature DCs would be a predictor of treatment response and outcome in metastatic RCC patients, who receive immunotherapy.*

The incidence and mortality of renal cell carcinoma (RCC) have been increasing in recent years in Japan (1). A quarter of patients with RCC have evidence of metastatic disease at the time of diagnosis. Metastases from RCC are resistant to chemotherapy and irradiation, and therefore various immunotherapeutic strategies using mainly interferon (IFN)-α or interleukin-2 (IL-2) have been evaluated for patients with advanced RCC (2-4). However, only a small minority of patients have experienced therapeutic benefits, while toxicity was severe and cost of treatment was high (5, 6). Determining predictive factors of treatment response would be helpful for selecting suitable patients for cytokine treatment and enhancing treatment benefit.

Considerable data is now available to help us predict the outcome for patients with advanced RCC receiving cytokine treatment. Performance status, number of organs with metastases, prior nephrectomy, degree of treatment-related thrombocytopenia, absence of prior IFN treatment, thyroid dysfunction, rebound lymphocytosis, erythropoietin production, and post-treatment elevations of tumor necrosis factor-α and IL-1 levels have been reported to associate with treatment response (7). RCC is considered to be an immunogenic tumor. This explains some of the responses to cytokine and other types of immune-based treatments and implies that the intratumoral milieu of immunity may be associated with the response to cytokine treatments.

The major histocompatibility (MHC) class II antigens serve as restriction elements for cells presenting antigens to CD-4 positive helper T-cells. It has been reported that most RCCs expressed MHC class II antigen, especially HLA-DR, on the tumor cells suggesting increased immunogenicity of the tumor and susceptibility to immune reaction of the host (8). Dendritic cells (DCs) are thought to participate in natural tumor immunity by migrating into tumors, where they acquire antigen, undergo activation and migrate to the lymph nodes to initiate the T-cell response against the tumor-associated antigens. A previous study has described the predominant infiltration of DCs, as well as T-cells and macrophages, into RCC, but not into benign adenomas, indicating an antitumor immune reaction (9). Tumor-associated macrophages (TAMs), which infiltrate into tumor tissues, tend to suppress cellular immunity *via* various cytokines (10) and promote tumor cell proliferation by facilitating angiogenesis (11), while activated macrophages outside the tumors should have cytotoxic effects against the tumor cells.

To address whether or not the local immune environment within the tumor might be associated with the outcome of cytokine treatment, RCC specimens collected from patients

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who received cytokine treatment were examined using immunohistochemistry for various immune-related or angiogenic parameters.

Patients and Methods

Patients. A total of 142 patients underwent nephrectomy between 1990 and 2000 at our institute. Twenty-eight patients received cytokine-based immunotherapy for metastases. Of these patients, 25 patients (20 men and 5 women) with an average age of 57.4±12.7, for whom sufficient follow-up information was available, were further evaluated. Metastases were present at nephrectomy in seven patients, and four weeks were required to elapse since prior nephrectomy before the treatment. The other 18 patients received the treatment when they developed metastatic diseases after nephrectomy. The average follow-up period after initiation of immunotherapy was 43.9±29.6 months. Thirteen patients had a single and twelve had multiple metastatic sites. The cytokine treatments consisted of interferon-α monotherapy in five cases and a combination of interferon-α, interleukin-2 and fluoropyrimidines-based chemotherapy in twenty cases.

Immunohistochemistry. The resected specimens were fixed in 4% paraformaldehyde solution and step-sectioned at 5 mm intervals, and then embedded in paraffin. Hematoxylin and eosin stained sections were used to select the most cellular part of the tumor. After thin sections (5 μm) from the selected areas were deparaffinized, antigen retrieval treatment was performed with a microwave oven in 0.01M sodium citrate buffer (pH 6.0). Endogenous peroxidase blocking was performed using 0.3% H_2O_2 for 30 minutes. Nonspecific binding was blocked by incubation with phosphate buffer saline containing 1% bovine serum albumin for 60 minutes. Primary antibodies were anti-CD83 antibody (Novocastra Laboratories, New Castle, UK) (diluted 1:20) and anti-S100 antibody (Dako Corporation, CA, USA) (diluted 1:500) for DCs, anti-CD8 antibody (Dako Cytomation, Denmark) (diluted 1:25), anti-HLA-DR antibody (Santa Crus Biotechnology, CA, USA) (diluted 1:200), anti-CD68 antibody (Dako Japan, Kyoto, Japan) (diluted 1:300) for TAMs, anti-factor-VIII related antigen (Dako Corporation, CA, USA) (diluted 1:500) for microvascular density (MVD) and anti-vascular endothelial growth factor (VEGF) antibody (Santa Crus Biotechnology, USA) (diluted 1:300). The sections were incubated with primary antibodies at 4C overnight followed by incubation with biotinylated secondary antibody and then with horseradish peroxidase conjugated avidin. Peroxidase was visualized using a liquid 3,3'-diaminobenzidine substrate kit and sections were counterstained with hematoxylin.

Semiquantitative evaluation for staining. All assessments of immunohistochemical staining were by light microscopy within the tumor. For the estimation of TAM density, entire sections were scanned at low magnification to identify hot spots where macrophages accumulated at high density. A minimum of ten fields including hot spots were examined at x400 magnification and the mean value of the CD68+ cells from the five most macrophage-rich fields was taken as the TAM density. The assessments of CD8+ T-cells and CD83+, S100+ DCs were performed likewise, and the mean values of the five highest counts were used for subsequent analyses. The HLA-DR status was evaluated in the tumor cells and not the mononuclear infiltrates. Estimated percentages of tumor cells stained for DR antigen, were grouped in an, grade 0 (0%), grade 1(1-25%), grade 2 (26-50%), grade 3 (51-75%) and grade 4 (76-100%) (12). At least, ten different fields were examined at x400 magnification and the highest grade was used for subsequent analysis. For MVD count, sections were scanned at low magnification to identify the 3 regions with the highest number of microvessels. The microvessels were counted in these areas at x200 magnification, and MVD was expressed as the mean number of vessels in these areas. For VEGF, the sections were examined at x200 magnification at a minimum of 10 fields. VEGF staining was semi-quantitatively assessed according to a 4-intensity scale (0: absence or faint membranous staining of rare tumor cells (<20%), +: membranous staining of most tumor cells (>50%), ++: diffuse membrane staining and cytoplasmic staining of minority of tumor cells (21-50%), and +++: significant cytoplasmic staining in most tumor cells (>50%), often associated with membranous reinforcement) (13).

Table I. *The comparison of the various parameters in RCC tumor tissue assessed by immunohistochemistry according to subsequent treatment response.*

Parameter	Responders (n=6)	Non-responders (n=19)	*p*-value
CD83	5.36±5.07	1.09±1.54	0.0126*
S100	6.80±6.21	1.94±1.63	0.0039*
CD8	54.72±86.99	76.63±94.91	0.8807*
HLA-DR	1.33±0.82	1.37±0.83	0.8719†
TAM	55.16±29.47	41.45±20.67	0.2134*
MVD	34.89±30.35	18.89±16.48	0.1063*
VEGF	1.66±0.81	1.63±0.89	0.7817†

*Student's *t*-test, †Mann-Whitney *U*-test. Numbers indicate the positive cell count for CD83, CD8, and MVD, and grade or intensity for HLA-DR and VEGF, expressed as mean±SD. TAM: tumor associated macrophage, MVD: microvascular density, VEGF: vascular endothelial growth factor.

Statistical analysis. In order to evaluate the feasibility of the above parameters as predictors of the treatment response, the patients were divided into two groups (high group and low group) based on the mean value of each parameter. The statistical endpoint was overall survival, which was measured from the commencement of the immunotherapy. The survival rate was estimated by the Kaplan-Meier method, and compared by the log-rank test. The Chi-square test was used to correlate treatment response with parameter status (high or low). Each independent data set was compared by the Student's *t*-test, or the Mann-Whitney *U*-test. Pearson's correlation coefficients were used to assess the various correlations. These analyses were performed using the Stat View 5.5 statistics program. The results were considered statistically significant when $p<0.05$.

Results

The overall response rate by immunotherapy was 24%. Namely, 6 out of 25 patients had an objective response with five complete responses (CR) and one partial response (PR), while seven of the remaining 19 patients had stable disease

Table II. *The comparison of the various parameters in RCC tumor tissue according to DCs status.*

	CD83			S100		
	High (n=8)	Low (n=17)	*p*-value	High (n=6)	Low (n=19)	*p*-value
CD8	49.04±84.99	81.88±95.44	0.4156*	43.72±85.79	80.10±94.09	0.4089*
HLA-DR	1.25±0.88	1.41±0.79	0.6314†	1.33±0.82	1.37±0.83	0.8719†
TAM	48.95±28.27	42.76±21.05	0.5445*	51.61±27.60	42.58±22.00	0.417*
MVD	33.50±26.02	17.66±16.88	0.0792*	28.72±25.37	20.84±19.94	0.4364*
VEGF	1.50±0.75	1.70±0.92	0.5618†	1.67±1.03	1.63±0.83	0.8625†

*Student's *t*-test, †Mann-Whitney *U*-test. Numbers indicate the positive cell count for CD83, CD8 and MVD, and grade or intensity for HLA-DR and VEGF, expressed as mean±SD. Patients were divided into 2 groups, either high or low, based on the mean value of the CD83 or S100 counts.

(SD) and twelve had progressive disease (PD). The responders (CR, PR) survived longer than nonresponders (SD, PD) with marginal significance ($p=0.052$). Consequently, 15 patients died until the end of follow-up period.

Table I shows the comparisons in the various parameters according to subsequent treatment response. No significant differences were observed in the scores of the TAM, CD8, HLA-DR, MVD, or VEGF, while the mean CD83 and S100 levels were significantly higher in responders ($p=0.0126$ and 0.0039, respectively). However, the scores of the various parameters did not differ significantly in relation to DC status (high or low) indicated by CD83 or S100 (Table II). A significant correlation was observed between CD83 and S100 status (high or low) and treatment response, that is, a higher CD83 and S100 status was associated with favorable treatment response ($p=0.036$ and 0.0151, respectively, Table III). However, only a higher CD83 status contributed to the survival outcome ($p=0.0339$, Figure 1).

Table IV shows the intercorrelation between TAM and the other parameters examined. No significant correlations were identified between them.

Discussion

To date, several clinical and laboratory factors predicting the outcome for patients with advanced RCC receiving cytokine treatments have been reported (7). The laboratory factors including thyroid dysfunction, thrombocytopenia or IL-1 production are treatment related events and thus not useful in patient selection for immunotherapy. The clinical factors such as absence of nephrectomy, presence of bone metastasis or liver metastasis, or poor performance status may help to identify patients whose life expectancy is poor even with systemic immunotherapy, they do not help to predict which patients are likely to respond to treatment. Given that treatment response remains a strong surrogate marker for clinical outcome, identifying the factors which can predict treatment response is of great interest.

Table III. *Case distribution stratified by parameter status and treatment response.*

Parameters	Responders (n=6)	Non-responders (n=19)	total	*p*-value (Chi-square test)
CD83				
High	4 (66.7%)	2 (33.3%)	6	
Low	2 (10.5%)	17 (89.5%)	19	0.0368
S100				
High	4 (66.7%)	2 (33.3%)	6	
Low	4 (21.1%)	15 (78.9%)	19	0.0151
CD8				
High	2 (33.3%)	4 (66.7%)	6	
Low	6 (31.5%)	13 (68.5%)	19	>0.999
HLA-DR				
High	1 (16.7%)	5 (83.3%)	6	
Low	4 (21.1%)	15 (78.9%)	19	>0.999
TAM				
High	4 (30.1%)	9 (69.9%)	13	
Low	2 (16.7%)	10 (83.3%)	12	0.6447
MVD				
High	2 (66.7%)	4 (33.3%)	6	
Low	4 (21.1%)	15 (78.9%)	19	0.3783
VEGF				
High	5 (31.3%)	11 (68.7%)	16	
Low	1 (11.1%)	8 (88.9%)	9	>0.999

Patients were divided into 2 groups, either high or low, based on the mean value of each parameter.

Table IV. *The correlation between TAM and the other parameters.*

	Rank	*p*-value*
TAM: CD83	–0.071	0.7407
TAM: S100	0.239	0.2518
TAM: CD8	–0.176	0.4052
TAM: HLA-DR	0.351	0.9057
TAM: MVD	0.032	0.0863
TAM: VEGF	0.253	0.2266

*Pearson's correlation co-eficients.

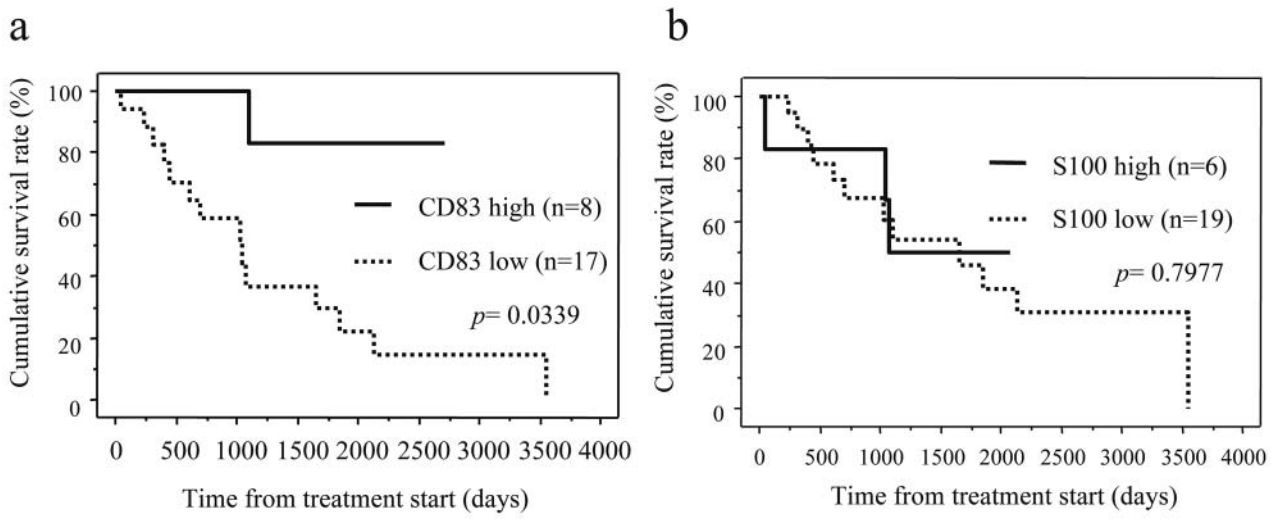

Figure 1. *Survival curve according to CD83 status (a) and S100 status (b). Patients with higher a number of CD83+ cells showed a significantly better survival than those with lower number.*

Information as to the histological or biological features of RCC which might predict of response to immunotherapy is limited. A strong trend favoring response in patients with clear cell carcinoma compared with those with variant tumors has been reported (14, 15).

Atkins *et al.* have demonstrated that high carbonic anhydrase IX expression associated with a favorable response to IL-2 treatment and longer survival (16).

Although RCC is considered to be an immunogenic tumor, there has been little investigation of the possibility that immunogenicity in tumors could predict treatment response. Rubin *et al.* have demonstrated that HLA-DR expression was observed in 58% (7/12) of primary RCC, but that DR status did not predict treatment response (12). Similarly, Mattijissen *et al.* have reported that no significant difference was found between responders and nonresponders with regard to HLA class I and II antigen expression (17). In agreement with these reports, the present study could not demonstrate any predictive significance of treatment response according to DR status, while HLA-DR expression was observed in all cases but one.

Dendritic cells are efficient and effective antigen-presenting cells which play a major role in initiating the primary immune response. They are the most potent stimulators of T-cell activation and are assumed to be essential in the antitumoral immune response (18, 19). Immunohistochemical studies using S100 staining have shown that an increased number of DCs within a tumor correlate with a better prognosis, in various types of tumor (20-24). In RCC a trend toward a better outcome and better prognosis following IFN treatment, with a higher number of S100+ DCs has also been found (25). Although S100 protein has long been used to indicate DCs, its functional implication is confusing (18, 19). Recently, CD83, a member of the immunoglobulin superfamily, has been shown to be expressed on activated and mature human DCs (26). Therefore, we used CD83 in addition to S100 as a functional DC marker to evaluate the correlation between DC infiltration in tumors and treatment outcome. Both CD83 and S100 status could predict treatment response. However, survival advantage was only found in the patients with a higher number of DCs when they were indicated by CD83 and not by S100. Thus, CD83 is likely to be a more reliable DC marker which reflects host immunity against malignancy.

The TAMs, which infiltrate into tumor tissue after differentiation from monocytes, produce various cytotoxic mediators, growth factors, cytokines, angiogenic factors and proteases depending on the microenvironment of the tumor tissue. Therefore, they play a complex role in the regulation of malignant progression and tumor growth since they have been shown to display both tumor promoting and inhibiting effects (10). TAMs within a tumor can actually work to suppress cellular immunity via various cytokines and promote tumor cell proliferation by facilitating angiogenesis, offering advantageous milieu for tumor growth (11). Although the results are conflicting concerning the role of TAMs in solid tumor tissue or the prognostic significance for patients, a recent report has demonstrated significant positive correlations for TAMs, microvascular density, and tumor proliferative index, with a tendency for higher parameter values to reflect poorer prognosis in RCC tumors (25). In the present study, The TAM status was not associated with either treatment response or survival outcome. Also, no significant correlations were identified between the TAMs and immune-related parameters

or angiogenic parameters. These negative results may be due to the particular population of cases analyzed in this study,v which all exhibited metastatic advanced disease.

Nonetheless, the DC status had an impact on both treatment response and prognosis in the metastatic RCC patients who received cytokine treatment. To our knowledge, this is the first report to show the clinical feasibility of CD83+, an activated and mature human DC marker, cell counting in tumor tissue for predicting treatment outcome for metastatic RCC patients treated by immunotherapy. Since the number of patients was relatively small, the present results and statistical trends need to be confirmed in a larger number of patients.

In conclusion, increased tumor infiltration of mature DCs in metastatic RCC patients who showed survival benefit from cytokine immunotherapy has been demonstrated, suggesting that antigen presentation is a critical factor in exerting antitumor effects by cytokine treatments. Patient selection for cytokine treatment based on the present results may help maximize treatment benefit.

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